Partial Rescue of Functional Interactions of a Nonpalmitoylated Mutant of the G-Protein $G\alpha_s$ by Fusion to the β-Adrenergic Receptor[†]

Ozlem Ugur,[*,‡] H. Ongun Onaran,[‡,§] and Teresa L. Z. Jones[||]

Department of Pharmacology and Clinical Pharmacology and Molecular Biology and Technology Research and Development Unit, Ankara University Faculty of Medicine, Sihhiye, 06100 Ankara, Turkey, and Metabolic Diseases Branch, National Institute of Diabetes and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received July 17, 2002; Revised Manuscript Received December 31, 2002

ABSTRACT: Most heterotrimeric G-protein α subunits are posttranslationally modified by palmitoylation, a reversible process that is dynamically regulated. We analyzed the effects of $G\alpha_s$ palmitoylation for its intracellular distribution and ability to couple to the β-adrenergic receptor (βAR) and stimulate adenylyl cyclase. Subcellular fractionation and immunofluorescence microscopy of stably transfected cyc$^-$ cells, which lack endogenous $G\alpha_s$, showed that wild-type $G\alpha_s$ was predominantly localized at the plasma membrane, but the mutant C3A-$G\alpha_s$, which does not incorporate [^{3}H]palmitate, was mostly associated with intracellular membranes. In agreement with this mislocalization, C3A-$G\alpha_s$ showed neither isoproterenol- or GTPγS-stimulated adenylyl cyclase activation nor GTPγS-sensitive high-affinity agonist binding, all of which were present in the wild-type $G\alpha_s$ expressing cells. Fusion of C3A-$G\alpha_s$ with the βAR [βAR-(C3A)$G\alpha_s$] partially rescued its ability to induce high-affinity agonist binding and to stimulate adenylyl cyclase activity after isoproterenol or GTPγS treatment. In comparison to results with the WT-$G\alpha_s$ and βAR (βAR-$G\alpha_s$) fusion protein, the βAR-(C3A)$G\alpha_s$ fusion protein was about half as efficient at coupling to the receptor and effector. Chemical depalmitoylation by hydroxylamine of membranes expressing βAR-$G\alpha_s$ reduced the high-affinity agonist binding and adenylyl cyclase activation to a similar degree as that observed in βAR-(C3A)$G\alpha_s$ expressing membranes. Altogether, these findings indicate that palmitoylation ensured proper localization of $G\alpha_s$ and facilitated bimolecular interactions of $G\alpha_s$ with the βAR and adenylyl cyclase.

Heterotrimeric G-proteins[1] play a crucial role at the plasma membrane, transducing extracellular signals of various kinds to intracellular effector molecules such as adenylyl cyclase, phospholipase C, and ion channels (*1*, *2*). Each heterotrimer consists of an α subunit, which exchanges GDP for GTP upon activation by its cognate receptor, and β and γ subunits, which are tightly bound together. In contrast to the receptors and effectors that are integral membrane proteins, G-protein subunits are peripheral membrane proteins associated with the inner face of the plasma membrane. The membrane attachment of Gα and Gγ subunits is facilitated by their covalent modification with lipids (*3*, *4*). Most Gα subunits undergo palmitoylation, the posttranslational attachment of palmitic acid through a labile thioester bond to amino-terminal cysteine residues. Palmitoylation is reversible, and palmitate turnover accelerates upon G-protein activation, suggesting a regulatory role for palmitoylation in G-protein signaling (*5*, *6*). However, the function of palmitoylation during G-protein signaling and particularly G_s signaling is not clearly understood. Does it act as a membrane-targeting signal or does it directly affect protein−protein interactions?

Site-directed mutagenesis to prevent palmitoylation on Gα subunits leads to defects in receptor and effector interactions (*7*−*15*), but mutation of cysteine residues, independent of the loss of palmitoylation, can alter receptor and effector interactions (*13*). In addition, depalmitoylation can reduce the effective concentration of Gα subunits at the plasma membrane (*15*−*17*). A decrement in plasma membrane localization makes evaluation of the role of palmitoylation in receptor and effector interactions difficult because these interactions depend on both the affinity and the relative plasma membrane concentrations of these proteins. To circumvent the problems of plasma membrane targeting, receptor−Gα fusion proteins have successfully been created that fuse the carboxy-terminal tail of the receptor with the amino terminus of the Gα subunit (reviewed in refs *18* and *19*). Fusion proteins of receptors with the nonpalmitoylated mutants of $G\alpha_{i1}$ and $G\alpha_{11}$ show a full restoration of receptor interactions for $G\alpha_{i1}$ and at least a partial restoration for $G\alpha_{11}$ (*10*, *20*). The effect of Gα palmitoylation on Gα−effector interactions was not tested in these studies (*10*, *20*). βAR-

[†] This work was supported in part by grants from the Turkish Scientific and Technical Research Council (SBAG-2105) and the Ankara University Research Fund (2001-08-09-054).

* To whom correspondence should be addressed. Tel: 90-312-310 6268. Fax: 90-312-310 6268. E-mail: mugur@dialup.ankara.edu.tr.

[‡] Department of Pharmacology and Clinical Pharmacology, Ankara University Faculty of Medicine.

[§] Molecular Biology and Technology Research and Development Unit, Ankara University Faculty of Medicine.

[||] National Institutes of Health.

[1] Abbreviations: G-protein, guanine nucleotide-binding protein; βAR, β-adrenergic receptor; WT, wild type; CYP, cyanopindolol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ERK, extracellular signal regulated kinase; SEM, standard error of the mean; AUC, area under curve.

10.1021/bi026470i CCC: $25.00
Published on Web 02/11/2003

$G\alpha_s$ fusion proteins have also been created but not previously used to test the function of $G\alpha_s$ palmitoylation. The βAR-$G\alpha_s$ fusion protein can undergo palmitoylation on both the βAR and $G\alpha_s$ (*21*), efficiently couple to adenylyl cyclase, maintain high- and low-affinity binding states, and show GTP turnover rates similar to rates obtained after reconstitution of purified proteins into phospholipid vesicles (*18*).

The goal of the present study was to determine the importance of palmitoylation on $G\alpha_s$ for its receptor and effector interactions. For this purpose, (1) we stably transfected S49 lymphoma cyc$^-$ cells, which do not express $G\alpha_s$, with wild-type (WT) or a nonpalmitoylated mutant of $G\alpha_s$ alone or as fusion proteins with the βAR, and (2) we treated membranes from the cells expressing the fusion proteins with hydroxylamine to remove the palmitate. We found that palmitoylation both targets $G\alpha_s$ to the plasma membrane and improves βAR$-G\alpha_s$ and $G\alpha_s-$adenylyl cyclase interactions independent of membrane targeting.

EXPERIMENTAL PROCEDURES

Materials. Cell culture media, fetal bovine serum, and antibiotics were purchased from Biochrom (Berlin, Germany). Enzymes used in molecular cloning were obtained from New England Biolabs (Beverly, MA). GTP, GTPγS, ATP, Pwo polymerase, and Complete Mini protease inhibitors were purchased from Roche Diagnostics (GmbH, Manheim, Germany). Isobutylmethylxanthine, hydroxylamine hydrochloride, forskolin, and (−)-isoproterenol were purchased from Sigma (Germany). Protein A−Sepharose CL-4B, [^{3}H]dihydroalprenolol, and [^{125}I]cyanopindolol were purchased from Amersham-Pharmacia (Buckinghamshire, England).

Plasmid Constructs and Mutagenesis. The cDNA encoding the long form of rat $G\alpha_s$ was in the pCDNA 3.1 (+) vector (Invitrogen, Carlsbad, CA). The C3A mutant of $G\alpha_s$ was generated using a site-directed mutagenesis method based on the polymerase chain reaction, QuikChange (Stratagene, La Jolla, CA), except the Pwo polymerase (Roche Diagnostics) was used. The cDNA encoding the 822 amino acid long human β_2-adrenergic receptor−rat $G\alpha_s$ fusion protein (βAR-$G\alpha_s$) in the pCDNA3 vector was a kind gift of Dr. Tommasso Costa (Istituto Superiore de Sanita, Roma, Italy). The same mutagenesis method was used to generate a palmitoylation-deficient fusion protein where the third residue on the $G\alpha_s$ portion, a cysteine, was replaced with alanine [βAR-(C3A)$G\alpha_s$]. Mutations were confirmed using ABI PRISM dye terminator cycle sequencing (Perkin-Elmer-Cetus, Norwalk, CT).

Cell Culture and Stable Transfection. S49 lymphoma cyc$^-$ cells were grown in Dulbecco's modified Eagle's media supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% (v/v) fetal bovine serum, at 37 °C in a humidified atmosphere of 5% CO_2. Cyc$^-$ cells were transfected by electroporation essentially as described before (*22*) except that transfections were performed with the Electroporator II (Invitrogen, San Diego, CA) at a setting of 500 V and 71 μF, and 40 μg of circular plasmid DNA was used for each transfection. Twenty-four hours after the transfection, cells were diluted with 20 mL of medium containing Geneticin (Gibco, Carlsbad, CA) at a final concentration of 700 μg/mL and distributed into 96-well plates in 0.2 mL aliquots. After 4 weeks, Geneticin-resistant clones were selected further according to their expression levels as assessed by immunoblotting [for the clones transfected with $G\alpha_s$ or (C3A)$G\alpha_s$] or isoproterenol-induced cAMP accumulation levels [for the clones transfected with the βAR-$G\alpha_s$ or βAR-(C3A)$G\alpha_s$]. Thus, clones expressing approximately equal amounts of $G\alpha_s$ or (C3A)$G\alpha_s$ in their membrane fractions and the βAR-$G\alpha_s$ or βAR-(C3A)$G\alpha_s$ clones having comparable isoproterenol-induced cAMP accumulation levels were selected and grown for further experiments.

Immunoblotting. Proteins were separated by SDS−PAGE, transferred to nitrocellulose paper, and detected with the polyclonal, affinity-purified RM antibody, which recognizes the carboxy-terminal decapeptide of $G\alpha_s$ (1 μg/mL) or a polyclonal antibody to the extracellular signal regulated kinase 2 (ERK2) (Santa Cruz Biotechnologies) (1:1000), and enhanced chemiluminesence (Amersham, Arlington Heights, IL) using horse radish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies (developed in M.B.T.R.D. Unit, Ankara University). An image analysis software (AIDA) and a camera system (Raytest, Straubenhardt, Germany) were used for the densitometric analyses of protein bands.

Immunocytochemistry and Confocal Microscopy. Since cyc$^-$ cells do not attach to glass or plastic surfaces even in the presence of extracellular matrix proteins, all the incubation and washing steps were performed by resuspending the cells in the indicated solutions followed by centrifugation at 200*g*. Otherwise, all of the procedures were essentially as described before (*23*). Briefly, (1−2) × 10^6 cells were washed three times with 1 mL of PBS at room temperature, fixed in 2% (w/v) paraformaldehyde in PBS for 20 min, and permeabilized with 0.1% Triton X-100 (v/v) in PBS for 15 min. After being blocked with 1% (w/v) BSA in PBS, cells were incubated for 1 h with 0.5 μg/mL RM antibody in PBS containing 0.1% BSA, followed by washing three times with 0.1% BSA in PBS. Cells were then incubated for 1 h with Cy3-labeled anti-rabbit antibody (ZYMED, San Francisco, CA) diluted 1:2000 in PBS containing 0.1% BSA. After being washed three times with PBS, cells were resuspended in 50 μL of PBS, pipetted onto a microscope slide, covered, and immediately visualized with a Zeiss LSM-510 confocal microscope (Germany) equipped with 543 He−Ne laser and 40× and 63× Zeiss plan-apo oil immersion objectives.

Cell Fractionation and Membrane Preparation. Cyc$^-$ cells, washed and pelleted in PBS, were lysed, homogenized, and fractionated into particulate and soluble fractions by centrifugation at 125000*g* for 1 h as described before, except mannitol was omitted from the homogenization buffer (*24*). For the adenylyl cyclase and binding assays, cells were lysed and homogenized by passing 10−15 times through a 26-gauge syringe tip in a buffer containing 5 mM Tris-HCl, pH 7.4, and a mixture of protease inhibitors, diluted according to manufacturer's recommendation (Complete Mini, Roche Diagnostics). Unbroken cells and nuclei were pelleted by centrifugation at 400*g* for 5 min. The membrane pellet, obtained by centrifugation for 1 h at 40000*g* of the supernatant, was washed once with a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, protease mixture, and 0.2 mg/mL dithiothreitol. The pellet was resuspended at a

concentration of 2−3 mg/mL in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, protease inhibitors, and 25% (w/v) sucrose and stored at −70 °C. Protein concentrations were determined by the Bradford assay using bovine serum albumin (BSA) as the standard (*25*).

Membrane Attachment after GTPγS Treatment. Sixty micrograms of cell membranes was incubated with and without 10 μM GTPγS in a buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM $MgCl_2$ for 4 h at 37 °C. The samples were separated into particulate and soluble fractions by centrifugation at 125000*g* for 1 h. The pellets were resuspended in the initial volume of buffer. Equal volumes of particulate and soluble fractions were subjected to SDS−PAGE for immunoblotting.

Determination of Membrane Adenylyl Cyclase Activity and cAMP Accumulation in Cells. Adenylyl cyclase activity in cell membranes was measured essentially as described before (*26*). Briefly, membranes were preincubated in 96-well plates (0.5−1 μg/well) with indicated stimulants for 5 min (or at least 1.5 h with GTPγS) in 100 μL, at 37 °C. Assay was initiated by adding 50 μL of adenylyl cyclase assay buffer (final concentrations: 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 1 mM isobutylmethylxanthine) and terminated after 5 min by adding 50 μL of 0.4 N HCl. For cAMP accumulation experiments in whole cells, cyc$^-$ cells were washed with serum-free media, incubated with 1 mM isobutylmethylxanthine for 15 min and with isoproterenol for an additional 15 min. Reaction was terminated by addition of 0.5 volume of 0.4 N HCl. The cAMP amount was measured by radioimmunoassay as described before (*26*).

Binding Experiments. Three micrograms of the membranes expressing WT- or C3A-$G\alpha_s$ or 1.5 μg of the membranes expressing β$_1$AR-$G\alpha_s$ or β$_1$AR-(C3A)$G\alpha_s$ was incubated in the presence of 20−40 pM [^{125}I]CYP and the indicated concentrations of isoproterenol, with or without GTPγS (10 μM) in the binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$) in a final volume of 250 μL, at 27 °C for 3 h. Pipettings were performed using a Biomek 2000 laboratory automation workstation (Beckman Instruments, Fullerton, CA). Reaction was stopped by filtration through Whatman GF/B filters with the use of a cell harvester (Skatron, Lier, Norway). Data were analyzed using the LIGAND program (*27*) or by the regression of the four-parameter logistic equation. GTPγS-induced shift in isoproterenol binding curves was analyzed by evaluating the area between the two binding curves obtained in the presence or absence of GTPγS. The area between the curves was obtained by subtracting the areas under the normalized binding curves, which were calculated according to Simpson's rule of numerical integration. Membrane amounts of the fusion proteins were determined by single point saturation binding assays, using 2 μg of membrane protein and 2 nM [^{125}I]CYP. Nonspecific binding was estimated in the presence of 10 μM propranolol. Whole cell saturation binding experiments were carried out by using [^{3}H]dihydroalprenolol as the radioligand at a concentration range of 0.003−100 nM (97 Ci/mmol specific activity). Cells that were washed with serum-free DMEM twice were incubated at 37 °C for 1 h, distributed to a 96-well plate at a concentration of 100000 cells per well, and incubated with indicated concentrations of [^{3}H]dihydroalprenolol in a final volume of 100 μL. Alprenolol at a concentration of 1 μM was used to determine the nonspecific binding for each concentration of [^{3}H]-dihydroalprenolol. After incubation at 37 °C for 120 min, cells were filtered through a Whatman GF/C filter, using a cell harvester. Radioactivity on the filter was counted by liquid scintillation by the use of a Wallac Microbeta Trilux counter.

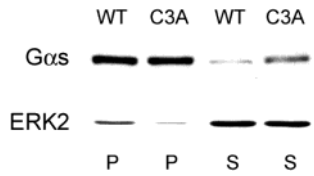

FIGURE 1: Subcellular fractionation of WT- and C3A-$G\alpha_s$. Cyc$^-$ cells, stably transfected with the WT- and C3A-$G\alpha_s$ cDNAs were separated into particulate (P) and soluble (S) fractions by centrifugation at 125000*g*, and 5 μg of protein from each fraction was subjected to SDS−PAGE. Proteins were analyzed by immunoblotting with the RM antibody specific for $G\alpha_s$ or an antibody specific for the cytosolic protein, ERK2.

Hydroxylamine Treatment. The pH of the hydroxylamine solution was adjusted to 7.4 with 100 mM Tris-HCl and KOH. The control solution contained 100 mM Tris-HCl and equimolar KCl. We observed that agonist binding characteristics of the β$_1$AR-$G\alpha_s$ fusion protein were sensitive both to the high salt concentration present in the matching control solution of 1 M hydroxylamine (usual concentration used to depalmitoylate proteins) and to the washing step that followed the incubation. Thus, we used 0.5 M hydroxylamine and omitted the washing step. Membranes were incubated in the solutions with or without hydroxylamine for 45 min at 20 °C, immediately diluted after the incubation, and added to the binding assay. The final hydroxylamine concentration in the assay was less than 2 mM. Hydroxylamine (1 M) or the corresponding control solution (see above) was used to treat membranes for the adenylyl cyclase assays.

RESULTS

Membrane Attachment and Intracellular Distribution of WT- and C3A-$G\alpha_s$. To assess the function and intracellular location of a nonpalmitoylated mutant of $G\alpha_s$, we expressed the WT and C3A mutant of $G\alpha_s$ by stable transfection of cyc$^-$ cells, which do not have endogenous $G\alpha_s$ (*28*). Mutation of the cysteine at residue 3 abolishes palmitoylation of $G\alpha_s$ (*7*, *24*, *29*). The cells were separated into crude membrane and cytosol fractions by centrifugation. ERK2, a cytosolic protein (*30*), was found predominantly in the soluble fraction with this fractionation procedure (Figure 1). Both the WT- and the C3A-$G\alpha_s$ were expressed primarily and approximately equally in the particulate fractions of the transfected cyc$^-$ cells with a 3-fold higher expression of C3A-$G\alpha_s$ in the soluble fraction (Figure 1).

Immunofluorescence confocal microscopy on the stably transfected cyc$^-$ cells showed that WT-$G\alpha_s$ was primarily localized at the plasma membrane, whereas C3A-$G\alpha_s$ was expressed within the cell (Figure 2). Essentially no staining was seen for the vector-transfected cells with the antibody specific for $G\alpha_s$ or for the transfected cells without the specific antibody (data not shown). This result, in conjunction with the fractionation studies showing C3A-$G\alpha_s$ predominantly in the particulate fraction (Figure 1), indicates that

Table 1: Functional Assays of Cyc$^-$ Cells Expressing the WT- and C3A-Gα_s[a]

	cAMP accumulation [pmol (10^6 cells)$^{-1}$ min^{-1}][b]	adenylyl cyclase activity [pmol (mg of protein)$^{-1}$ min^{-1}][c]		isoproterenol binding K_d (nM)[d]		
				GTPγS (−)		
	isoproterenol (100 μM)	isoproterenol (100 μM)	GTPγS (100 μM)	K_H	K_L	GTPγS (+)
vector	0.001 ± 0.006	5.1 ± 0.3	11.8 ± 1.7			
C3A-Gα_s	0.42 ± 0.04	4.5 ± 1.2	20.3 ± 0.1		116 ± 62.9	163 ± 64.7
WT-Gα_s	2.6 ± 0.02	59.4 ± 16.1	135.7 ± 32	4.4 ± 0.3[e]	566 ± 163	156 ± 95.4

[a] Cyc$^-$ cells were stably transfected with vector alone or with the cDNAs for the WT or C3A mutant of Gα_s. [b] cAMP accumulation assays were performed in whole cells after preincubation in 1 mM isobutylmethylxanthine for 15 min and then with and without isoproterenol for 15 min. Shown are the net isoproterenol-induced accumulations, i.e., the difference between the values with and without isoproterenol treatment. Data are the mean ± SEM from two experiments performed in six replicates. [c] The adenylyl cyclase assay was performed on membranes preincubated for 5 min with isoproterenol and 1 μM GTP or for 90 min with GTPγS and incubated in the assay buffer for 5 min as described under Experimental Procedures. Shown are the net activities, i.e., the difference between the values with and without the indicated treatments. Data are the mean ± SEM from two experiments. [d] The competition binding assays were performed as described under Experimental Procedures on membranes incubated with 20 pM [^{125}I]CYP and a range of isoproterenol concentrations in the presence or absence of 10 μM GTPγS for 3 h. Binding parameters were estimated by the use of the LIGAND program. K_H and K_L are the high- and low-affinity equilibrium dissociation constants for isoproterenol, respectively. Data are the mean ± SEM for two experiments. [e] The percentage of high-affinity binding sites was 54 ± 16%.

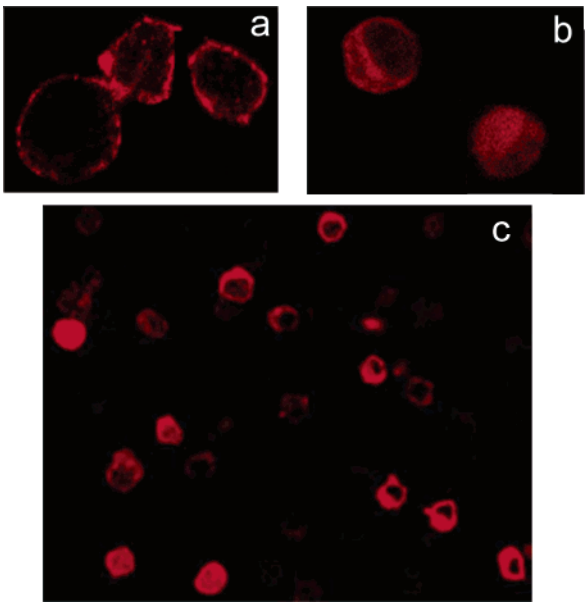

FIGURE 2: Intracellular localization of WT- and C3A-Gα_s in stably transfected cyc$^-$ cells. Cells expressing WT-Gα_s (a) and C3A-Gα_s (b, c) were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained by incubations first with the rabbit polyclonal RM antibody to Gα_s and subsequently with the CY3-labeled anti-rabbit antibody. A confocal microscope was used to examine the cells.

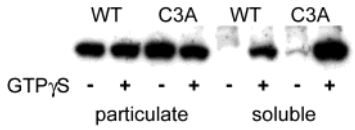

FIGURE 3: GTPγS-induced release of Gα_s from the membrane. Equal amounts of membranes expressing WT- or C3A-Gα_s were incubated with or without 10 μM GTPγS at 37 °C for 4 h and separated into particulate and soluble fractions by centrifugation at 100000*g* for 1 h. Pellets were resuspended in the initial volume of buffer, and equal volumes from each fraction were subjected to immunoblotting with an antibody to Gα_s.

mutation of C3 led to a loss of the specific targeting of Gα_s to the plasma membrane but that the C3A mutant can still nonspecifically attach to intracellular membranes.

C3A-Gα_s Cannot Functionally Interact with the βAR or Adenylyl Cyclase. We tested the ability of the nonpalmitoylated C3A-Gα_s to couple to the βAR and adenylyl cyclase. In a whole cell assay, the C3A-Gα_s transfected cells showed a minimal increase in cAMP accumulation in response to isoproterenol compared to the response in the WT-Gα_s transfected cells (Table 1). The adenylyl cyclase activity in response to isoproterenol or GTPγS of membranes from cells expressing the nonpalmitoylated C3A mutant was concentration-independent (data not shown) and did not differ (isoproterenol) or differed only marginally (GTPγS) from results from membranes from vector-transfected cells (Table 1). In comparison, the cells expressing the WT-Gα_s showed a prominent and concentration-dependent increase in adenylyl cyclase activity after incubation with isoproterenol or GTPγS (Table 1; only the net isoproterenol- or GTPγS-induced activities are shown).

We studied the agonist binding characteristics of the βAR in membranes from WT- or C3A-Gα_s transfected cells because the presence of high-affinity agonist binding and its sensitivity to a guanine nucleotide reflect the functional interaction between a receptor and a G-protein. The membranes from cells expressing the WT-Gα_s showed high- and low-affinity agonist binding, and GTPγS abolished the high-affinity component of the binding (Table 1). In membranes expressing C3A-Gα_s, isoproterenol exhibited single-site binding in both the absence and presence of GTPγS (Table 1). The C3A-Gα_s mutant was able to bind GTPγS because incubation of membranes from the stably transfected cells with GTPγS showed a release of the C3A-Gα_s mutant to the soluble fraction that was considerably higher compared to the release of WT-Gα_s or C3A-Gα_s without GTPγS (Figure 3). These results show that the nonpalmitoylated C3A-Gα_s did not functionally interact with its receptor or effector.

Expression of the βAR-Gα_s Fusion Proteins. We assessed the role of Gα_s palmitoylation for βAR−G$_s$ and G$_s$−adenylyl cyclase interactions independently from its role in membrane attachment and trafficking by using a βAR-Gα_s fusion protein (Figure 4A). This fusion protein ensures a 1:1 stoichiometry for βAR and Gα_s at the membrane, regardless of the trafficking properties of the Gα_s. The βAR-Gα_s fusion protein can undergo palmitoylation through a thioester linkage to residues corresponding to C341 in the βAR and C3 in Gα_s (*21*). (We were unsuccessful in immunoprecipitating adequate quantities of the fusion protein in cyc$^-$ cells to detect [^{3}H]palmitate incorporation.) Both the βAR-Gα_s and the βAR-(C3A)Gα_s cells were expressed as approxi-

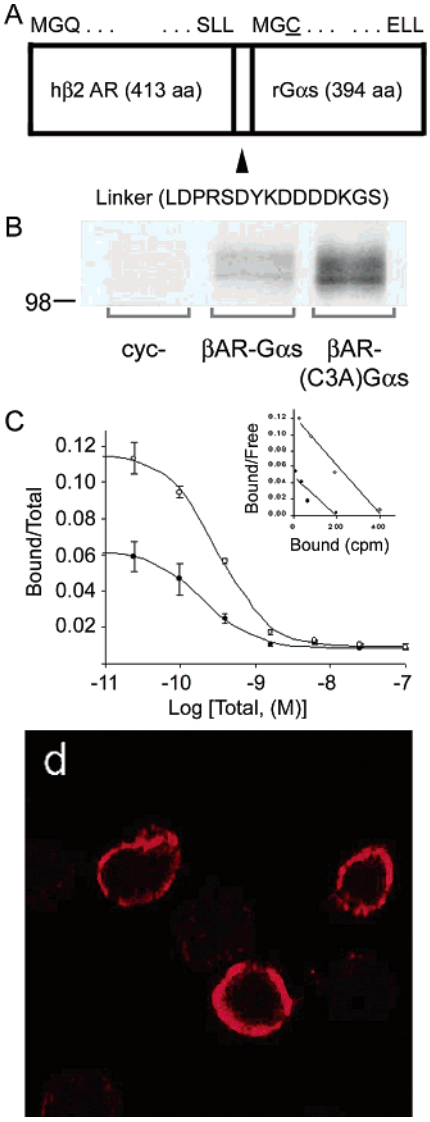

FIGURE 4: (A) Schematic representation of the fusion protein of human β_2-adrenergic receptor ($h\beta_2AR$) and the long form of rat $G\alpha_s$ ($rG\alpha_s$). (B) Expression of βAR-$G\alpha_s$ and βAR-(C3A)$G\alpha_s$ in stably transfected cyc$^-$ cell membranes. Ten micrograms of membrane protein from cyc$^-$ cells or cells stably transfected with the indicated cDNAs was subjected to SDS−PAGE and immunoblotting with an antibody specific for the carboxy terminus of $G\alpha_s$. The migration of the 98 kDa molecular mass marker is shown to the left. (C) Cell surface expression levels of fusion proteins. Cyc$^-$ cells expressing βAR-$G\alpha_s$ (●) or βAR-(C3A)$G\alpha_s$ (○) were incubated with the indicated concentrations of [^{3}H]dihydroalprenolol for 2 h at 37 °C and filtered through a Whatman GF/C filter. The first four data points at lower concentrations are presented in a Scatchard graph (inset). Estimated expression levels were 10000 and 20000 copies per cell for βAR-$G\alpha_s$ and βAR-(C3A)$G\alpha_s$, respectively. (D) Intracellular localization of βAR-$G\alpha_s$. Cells expressing βAR-$G\alpha_s$ were fixed in 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained by incubations first with the rabbit polyclonal RM antibody and subsequently with the CY3-labeled anti-rabbit antibody. A confocal microscope was used to examine the cells.

mately 105 kDa proteins in stably transfected cyc$^-$ cells as seen by immunoblotting with an antibody to the carboxy-terminal sequence of $G\alpha_s$ (Figure 4B). Their expression levels as assessed by [^{125}I]CYP saturation binding were 4.9 and 8.0 pmol/mg of membrane protein for the βAR-$G\alpha_s$ and βAR-(C3A)$G\alpha_s$ cells, respectively, that correlates with the higher expression level of the βAR-(C3A)$G\alpha_s$ seen on immunoblotting. Scatchard analysis of the saturation [^{3}H]-dihydrolprenolol binding experiments conducted on intact cells showed about a 2-fold higher cell surface expression of the βAR-(C3A)$G\alpha_s$ compared to the βAR-$G\alpha_s$ (Figure 4C). The different levels of expression were likely due to selecting clones with similar cAMP accumulation after isoproterenol stimulation (see below). The cell surface expression of the βAR-$G\alpha_s$ was also seen by immunofluorescence microscopy using an antibody to the carboxy terminus of $G\alpha_s$ (Figure 4D). The pattern of staining at the cell periphery for the receptor−$G\alpha_s$ fusion protein was similar to the staining for the WT-$G\alpha_s$ (Figure 2a).

Receptor and Effector Coupling of the βAR-(C3A)$G\alpha_s$ Fusion Protein. Cyc$^-$ cell membranes expressing the βAR-$G\alpha_s$ exhibited GTPγS-sensitive, high-affinity isoproterenol binding, indicating a functional coupling of these proteins (Figure 5A). This high-affinity isoproterenol binding was present in the membranes expressing the βAR-(C3A)$G\alpha_s$ as well but was reduced compared to the membranes expressing βAR-$G\alpha_s$. As a rough measure of GTPγS-induced shift in agonist binding curves, we used the area between the binding curves obtained in the presence or absence of GTPγS. This quantity depends on the relative positions of the two binding curves and was calculated as the difference between the whole areas under the two individual normalized binding curves (denoted as ΔAUC). The ΔAUC calculated from the isoproterenol binding curves of βAR-(C3A)$G\alpha_s$ expressing membranes was about one-third of that obtained with the membranes expressing the βAR-$G\alpha_s$ (Figure 5B).

Consistent with the binding data, isoproterenol-stimulated adenylyl cyclase activity of the membranes expressing the βAR-(C3A)$G\alpha_s$ was reduced about 45% compared to the membranes expressing βAR-$G\alpha_s$ (Figures 6A and 7A). Maximum GTPγS-induced adenylyl cyclase activity of βAR-(C3A)$G\alpha_s$ was also reduced by about 40% compared to βAR-$G\alpha_s$ (Figures 6B and 7B). The higher basal activity in the isoproterenol assay compared to the GTPγS assay was likely due to the presence of GTP in the assay buffer with isoproterenol. These results demonstrate that the functional interactions of C3A-$G\alpha_s$ with the βAR or adenylyl cyclase were less efficient compared to WT-$G\alpha_s$ when both were expressed as fusion proteins with the βAR.

Hydroxylamine Treatment of Membranes from Cells Expressing the βAR-$G\alpha_s$ Fusion Protein. To determine if our results with the βAR-(C3A)$G\alpha_s$ fusion protein were due to mutation of the cysteine residue rather than the loss of palmitoylation, we performed functional studies on cell membranes that had been treated with hydroxylamine to break thioester bonds and release palmitate. Both partners of the βAR-$G\alpha_s$ fusion protein can be depalmitoylated upon treatment with hydroxylamine (*21*). So, a possible confounding factor in our experiments could be the removal of palmitate on the βAR. However, depalmitoylation of the βAR by hydroxylamine treatment and then reconstitution into a membrane preparation did not change adenylyl cyclase activity after isoproterenol stimulation, indicating that the ligand binding and G-protein coupling characteristics of the nonpalmitoylated βAR remain intact (*31*).

Hydroxylamine treatment of membranes from cells expressing βAR-$G\alpha_s$ reduced the GTPγS-induced shift in isoproterenol binding by about 50% compared to membranes incubated in the control solution (Figure 5C). Treatment of

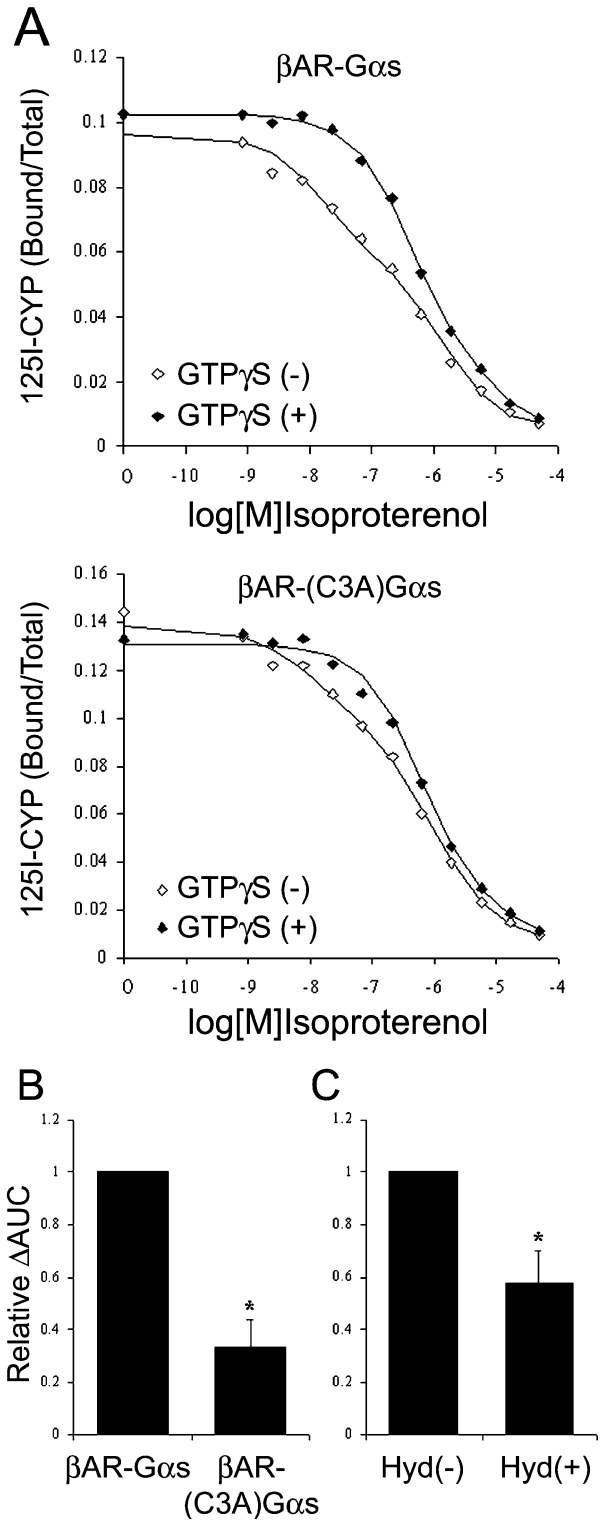

FIGURE 5: GTPγS-induced shift in isoproterenol binding in cyc$^-$ cell membranes. (A, B) An isoproterenol−[^{125}I]CYP competition binding assay was performed as described in Table 1 for cyc$^-$ cell membranes expressing βAR-Gα_s and βAR-(C3A)Gα_s or (C) in membranes expressing βAR-Gα_s, treated with or without 0.5 M neutral hydroxylamine (Hyd). (B, C) The area between the normalized binding curves obtained in the presence or absence of GTPγS (ΔAUC) was calculated as described under Experimental Procedures. Mean ΔAUCs (±SEM) of five to six experiments are presented relative to βAR-Gα_s in (B) and relative to Hyd (−) in (C). The asterisk notes significant differences with $p < 0.05$ on a paired *t*-test.

membranes with hydroxylamine decreased the isoproterenol- and GTPγS-stimulated adenylyl cyclase activity of the βAR-Gα_s fusion protein by 60% and 55%, respectively, to levels similar to corresponding activities of both the hydroxylamine-treated and untreated βAR-(C3A)Gα_s expressing membranes (Figure 7).

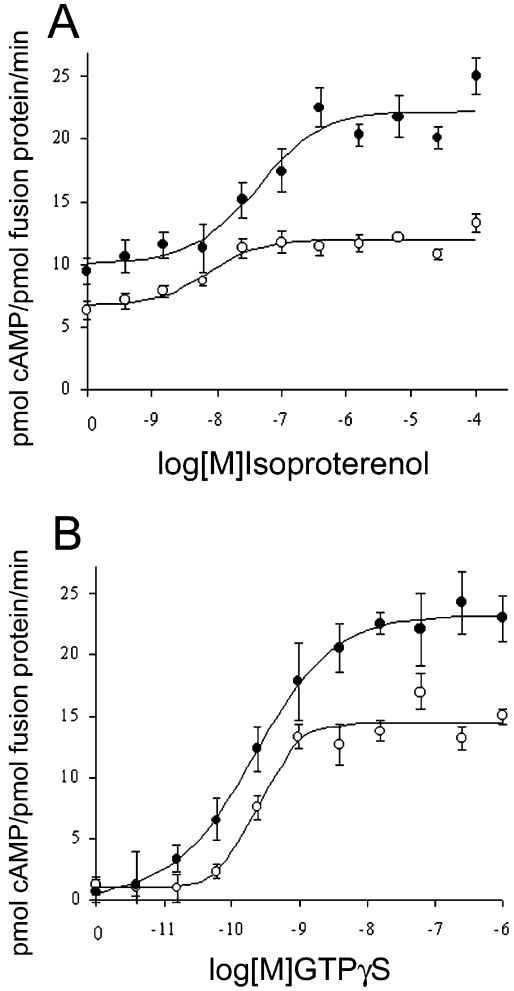

FIGURE 6: Isoproterenol- or GTPγS-stimulated adenylyl cyclase activity of cyc$^-$ cell membranes expressing the fusion proteins. (A) Membranes expressing βAR-Gα_s (●) or βAR-(C3A)Gα_s (○) were preincubated for 5 min with the indicated concentrations of isoproterenol. Adenylyl cyclase assay was initiated by the addition of assay buffer containing 1 μM GTP. (B) Membranes were preincubated with the indicated concentrations of GTPγS for 90 min, and then the adenylyl cyclase assay was performed in the presence of GTPγS. Shown are the representatives of two experiments performed in quadruplicate. A four-parameter logistic equation was fit to the data.

Forskolin-stimulated adenylyl cyclase activities of the membranes expressing βAR-Gα_s and βAR-(C3A)Gα_s were reduced by about 25% and 35%, respectively, after hydroxylamine treatment (Figure 8), possibly from a direct effect of hydroxylamine on adenylyl cyclase (*32*). However, the excess 35% and 30% decrease seen after hydroxylamine treatment in isoproterenol- and GTPγS-stimulated adenylyl cyclase activities, respectively, of the βAR-Gα_s expressing membranes was absent in βAR-(C3A)Gα_s membranes (Figure 8).

Hydroxylamine can have nonspecific effects including changes in membrane viscosity, but the lack of further functional impairment of the βAR-(C3A)Gα_s mutant beyond the nonspecific effects on adenylyl cyclase suggests that the primary effect of hydroxylamine in these experiments was depalmitoylation of Gα_s. These results, in conjunction with

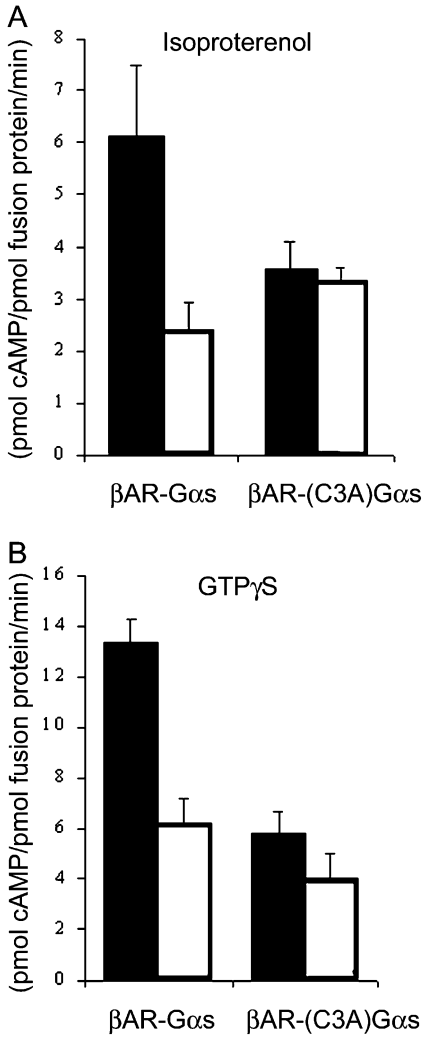

FIGURE 7: Effect of hydroxylamine treatment on isoproterenol- or GTPγS-stimulated adenylyl cyclase activity of cyc$^-$ cell membranes expressing the fusion proteins. Membranes were treated with (white bars) or without (black bars) 1 M hydroxylamine at room temperature for 45 min and assayed for isoproterenol-induced (A) or GTPγS-induced (B) adenylyl cyclase activity. The adenylyl cyclase assay was performed exactly as described for Figure 6. For (A), the adenylyl cyclase activity shown is the difference between the activity in the presence of 100 μM isoproterenol + 1 μM GTP and the activity in the presence of 1 μM GTP. For (B) GTPγS was used at a concentration of 1 μM. Data are the mean ± SEM of four experiments performed in quadruplicate.

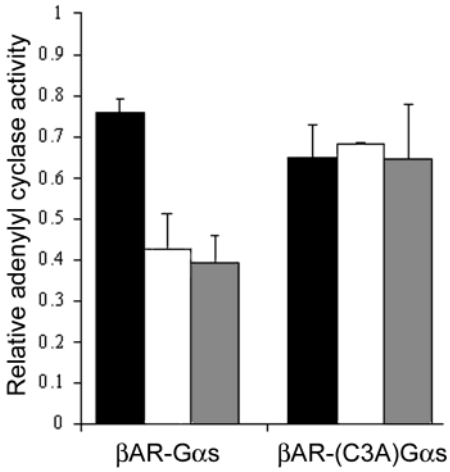

FIGURE 8: Effect of hydroxylamine treatment on the adenylyl cyclase activity of cyc$^-$ cell membranes expressing the fusion proteins. Membranes were preincubated with or without 1 M hydroxylamine at room temperature for 45 min and assayed for forskolin-stimulated (black bars), isoproterenol-stimulated (white bars), or GTPγS-stimulated (gray bars) adenylyl cyclase activity as described for Figure 6. Forskolin, isoproterenol, and GTPγS concentrations were 0.1 mM, 100 μM, and 1 μM, respectively. Adenylyl cyclase activities of hydroxylamine-treated membranes are presented relative to the control membranes. Data are the mean ± SEM of four experiments performed in quadruplicate.

those using the βAR-(C3A)Gα_s fusion protein, indicate that localizing a nonpalmitoylated Gα_s at the plasma membrane could restore some of its signaling ability. However, palmitoylation was still necessary for Gα_s to couple to the βAR and adenylyl cyclase with full efficiency.

DISCUSSION

Cells control protein palmitoylation to regulate the membrane attachment, intracellular targeting, and conformation of proteins. Here we dissected the role of palmitoylation on protein interactions for Gα_s. We found that Gα_s needed palmitoylation for plasma membrane localization. Forcing Gα_s to the plasma membrane through fusion of a nonpalmitoylated mutant of Gα_s to the βAR only partially rescued its ability to functionally couple to the βAR and adenylyl cyclase. This result is unlikely to be secondary to the cysteine point mutation because removal of palmitate from WT-Gα_s showed similar results: compromised formation of a receptor−G-protein complex and poor stimulation of adenylyl cyclase.

Palmitoylation Supports Plasma Membrane Localization of Gα_s. G-protein signaling occurs at the plasma membrane where G-proteins transduce signals from cell surface receptors to membrane-bound effectors. Palmitoylation is a critical factor in this localization of G-proteins. Mutation of amino-terminal cysteine residues that prevent palmitoylation disrupts the plasma membrane localization of some Gα subunits (*15*−*17*). The intracellular location of the C3A mutant of Gα_s could not be determined in a previous study because it was predominantly soluble (*7*, *17*). Our results show that the plasma membrane targeting of Gα_s was disrupted with mutation of the acylation site. The inability of the nonpalmitoylated C3A-Gα_s to talk to its receptor and effector was likely due to both decreased plasma membrane localization and the diminished protein interactions of this mutant.

Protein acyl transferase activity (*33*) and G$\beta\gamma$ subunits are found at the plasma membrane (*34*), where they regulate reciprocally the localization of Gα_s: G$\beta\gamma$ binding is needed for palmitoylation (*17*), and palmitoylation increases the affinity of the Gα_s subunit for G$\beta\gamma$ (*35*). In addition, the hydrophobicity of palmitoylation efficiently stops translocation off the membrane (*36*). For the "dock and lock" model of Gα targeting, G$\beta\gamma$ binding docks the Gα subunit, and palmitoylation locks it in place (*37*). Therefore, our finding that the C3A mutant was located on membranes besides the plasma membrane suggests that a protein interaction besides G$\beta\gamma$ binding or another modification may be involved. Strong experimental evidence points to the presence of a second hydrophobic modification near the amino terminus of Gα_s, though it has not been identified, yet (*38*). This factor alone may allow weak nonspecific membrane attachment whose detection would be sensitive to experimental conditions such as epitope tagging and methods of depalmito-

ylation (mutational vs chemical and enzymatic) and explain the wide range of reported loss of $G\alpha_s$ membrane attachment induced by depalmitoylation (*7*, *24*, *29*, *39*).

Palmitoylation of $G\alpha_s$ and Receptor and Effector Interactions. Palmitoylation is not an absolute requirement for G_s signaling because recombinant $G\alpha_s$ purified from bacteria is not palmitoylated and can couple to the βAR and adenylyl cyclase after reconstitution into membranes (*40*). Our results show that placing a nonpalmitoylated $G\alpha_s$ close enough to the βAR and adenylyl cyclase by fusing $G\alpha_s$ to the carboxy-terminal tail of the βAR was adequate to restore some of G_s signaling. However, even with $G\alpha_s$ residing on the correct membrane, the lack of palmitoylation still limited receptor and effector coupling.

Impairment in functional $G\alpha_s$−receptor and $G\alpha_s$−effector coupling could not occur through release of $G\alpha_s$ from the membrane because it was still fused to the receptor but could occur through changes on $G\alpha_s$ relating to its (1) conformation, (2) proximity to the membrane, or (3) orientation to the membrane and membrane-bound proteins. These mechanisms are not mutually exclusive. Palmitoylation can also target proteins, including G-proteins, to membrane microdomains enriched in cholesterol and sphingolipids (*41*). However, the functional significance of this targeting for G_s signaling (*42*) and whether the βAR-$G\alpha_s$ fusion protein is in these microdomains is not clear. The juxtaposition of $G\alpha_s$ at the membrane in a limited range of orientations may be necessary for $G\alpha_s$ to nestle in the intracellular loops of the receptor and effector. The addition of a myristoylation signal to the amino terminus of a nonpalmitoylated mutant of $G\alpha_s$ can restore its effector coupling (*7*). The ability of the nonpalmitoylated $G\alpha_{i1}$ to efficiently couple to the receptor after fusion to the α_{2A}-adrenoreceptor (*20*) may be explained by a closer proximity to the membrane. The carboxy-terminal tail of the α_{2A}-adrenoreceptor has only 24 residues, whereas the βAR has 86 residues and an additional 15 residues in our construct. The crystal structure of $G\alpha_s$ binding to the catalytic domains of adenylyl cyclase (*43*) and a model of G-protein coupling to the receptor, based on the crystal structure of rhodopsin and transducin (*44*), highlight the importance of proper orientation and distance of $G\alpha_s$ to the membrane for these interactions.

In the present study, we did not address the interaction between $G\alpha_s$ and the $G\beta\gamma$ complex. Palmitoylation on $G\alpha_s$ can increase its affinity for $G\beta\gamma$ (*35*), and a decrease in heterotrimer formation could decrease the receptor coupling of G_s. However, $G\beta\gamma$ complexes may be unnecessary for high-affinity agonist binding to the receptor because overexpression of $G\beta\gamma$ subunits in Sf9 cells did not increase the ternary complex formation of the βAR-$G\alpha_s$ fusion protein (*45*). Clearly, this point needs further clarification. We also did not address palmitate turnover on the βAR-$G\alpha_s$ fusion protein. Within a membrane preparation, palmitate turnover may not occur because an identified acyl protein thioesterase, APT1, is a cytosolic protein (*46*). Our results could overemphasize the importance of palmitoylation, if persistent palmitoylation of $G\alpha_s$ improves its activity. If depalmitoylation does occur during activation or preparation of the membranes, then our results would underestimate the effect of palmitoylation because the stoichiometry of palmitoylation on $G\alpha_s$, which is quite high in the cell (*47*), could be lower depending on the enzyme activities of the thioesterase and transferase in the membrane.

Agonist binding to a G_s-coupled receptor starts both a GTPase and thioacylation cycle whose actions finely regulate $G\alpha_s$ signaling within the cell. The novel finding of this study is that palmitoylation may have a role in the functional interactions of $G\alpha_s$ with both the βAR and adenylyl cyclase, in addition to localizing $G\alpha_s$ at the plasma membrane. The question of whether palmitoylation of $G\alpha_s$ modifies βAR and adenylyl cyclase interactions through a change in protein conformation or stable anchorage in a proper orientation remains to be answered.

ACKNOWLEDGMENT

We thank Dr. Alp Can (Histology and Embryology Department, Ankara University Faculty of Medicine) for assistance with the confocal microscopy, Dr. Yukiko Miura for help with the cyc^- cells, and Dr. Graeme Milligan for critical reading of the manuscript.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
2. Hamm, H. E. (1998) *J. Biol. Chem. 273*, 669−672.
3. Casey, P. J. (1995) *Science 268*, 221−225.
4. Wedegaertner, P. B. (1998) *Biol. Signals Recept. 7*, 125−135.
5. Mumby, S. M. (1997) *Curr. Opin. Cell Biol. 9*, 148−154.
6. Dunphy, J. T., and Linder, M. E. (1998) *Biochim. Biophys. Acta 1436*, 245−261.
7. Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., and Bourne, H. R. (1993) *J. Biol. Chem. 268*, 25001−25008.
8. Edgerton, M. D., Chabert, C., Chollet, A., and Arkinstall, S. (1994) *FEBS Lett. 354*, 195−199.
9. Wise, A., Grassie, M. A., Parenti, M., Lee, M., Rees, S., and Milligan, G. (1997) *Biochemistry 36*, 10620−10629.
10. Stevens, P. A., Pediani, J., Carrillo, J. J., and Milligan, G. (2001) *J. Biol. Chem. 276*, 35883−35890.
11. Ponimaskin, E., Harteneck, C., Schultz, G., and Schmidt, M. F. (1998) *FEBS Lett. 429*, 370−374.
12. Ponimaskin, E., Behn, H., Adarichev, V., Voyno-Yasenetskaya, T. A., Offermanns, S., and Schmidt, M. F. (2000) *FEBS Lett. 478*, 173−177.
13. Hepler, J. R., Biddlecome, G. H., Kleuss, C., Camp, L. A., Hofmann, S. L., Ross, E. M., and Gilman, A. G. (1996) *J. Biol. Chem. 271*, 496−504.
14. Jones, T. L. Z., and Gutkind, J. S. (1998) *Biochemistry 37*, 3196−3202.
15. Bhattacharyya, R., and Wedegaertner, P. B. (2000) *J. Biol. Chem. 275*, 14992−14999.
16. Morales, J., Fishburn, C. S., Wilson, P. T., and Bourne, H. R. (1998) *Mol. Biol. Cell 9*, 1−14.
17. Evanko, D. S., Thiyagarajan, M. M., Siderovski, D. P., and Wedegaertner, P. B. (2001) *J. Biol. Chem. 276*, 23945−23953.
18. Seifert, R., Wenzel-Seifert, K., and Kobilka, B. K. (1999) *Trends Pharmacol. Sci. 20*, 383−389.
19. Milligan, G. (2000) *Trends Pharmacol. Sci. 21*, 24−28.
20. Wise, A., and Milligan, G. (1997) *J. Biol. Chem. 272*, 24673−24678.
21. Loisel, T. P., Ansanay, H., Adam, L., Marullo, S., Seifert, R., Lagace, M., and Bouvier, M. (1999) *J. Biol. Chem. 274*, 31014−31019.
22. Gonzales, J. M., O'Donnell, J. K., Stadel, J. M., Sweet, R. W., and Molinoff, P. B. (1992) *J. Neurochem. 58*, 1093−1103.
23. Ugur, O., and Jones, T. L. Z. (2000) *Mol. Biol. Cell 11*, 1421−1432.
24. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. Z. (1993) *Biochemistry 32*, 8057−8061.
25. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
26. Ugur, O., and Onaran, H. O. (1997) *Biochem. J. 323*, 765−776.
27. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.
28. Bourne, H. R., Beiderman, B., Steinberg, F., and Brothers, V. M. (1982) *Mol. Pharmacol. 22*, 204−210.

29. Mumby, S. M., Kleuss, C., and Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2800−2804.
30. Hochholdinger, F., Baier, G., Nogalo, A., Bauer, B., Grunicke, H. H., and Uberall, F. (1999) *Mol. Cell. Biol. 19*, 8052−8065.
31. Moffett, S., Adam, L., Bonin, H., Loisel, T. P., Bouvier, M., and Mouillac, B. (1996) *J. Biol. Chem. 271*, 21490−21497.
32. Mollner, S., Beck, K., and Pfeuffer, T. (1995) *FEBS Lett. 371*, 241−244.
33. Dunphy, J. T., Greentree, W. K., Manahan, C. L., and Linder, M. E. (1996) *J. Biol. Chem. 271*, 7154−7159.
34. Denker, S. P., McCaffery, J. M., Palade, G. E., Insel, P. A., and Farquhar, M. G. (1996) *J. Cell Biol. 133*, 1027−1040.
35. Iiri, T., Backlund, P. S., Jr., Jones, T. L. Z., Wedegaertner, P. B., and Bourne, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14592−14597.
36. Yang, S., Zhang, L., and Huang, Y. (2001) *FEBS Lett. 498*, 76−81.
37. Fishburn, C. S., Pollitt, S. K., and Bourne, H. R. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 1085−1090.
38. Kleuss, C., and Gilman, A. G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6116−6120.
39. Huang, C., Duncan, J. A., Gilman, A. G., and Mumby, S. M. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 412−417.
40. Graziano, M. P., Freissmuth, M., and Gilman, A. G. (1989) *J. Biol. Chem. 264*, 409−418.
41. Resh, M. D. (1999) *Biochim. Biophys. Acta 1451*, 1−16.
42. Miura, Y., Hanada, K., and Jones, T. L. Z. (2001) *Biochemistry 40*, 15418−15423.
43. Tesmer, J. J., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) *Science 278*, 1907−1916.
44. Hamm, H. E. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 4819−4821.
45. Seifert, R., Lee, T. W., Lam, V. T., and Kobilka, B. K. (1998) *Eur. J. Biochem. 255*, 369−382.
46. Duncan, J. A., and Gilman, A. G. (1998) *J. Biol. Chem. 273*, 15830−15837.
47. Jones, T. L. Z., Degtyarev, M. Y., and Backlund, P. S., Jr. (1997) *Biochemistry 36*, 7185−7191.

BI026470I